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Effect of Silicate Grain Shape, Structure, and Location on the Biomass and Community Structure of Colonizing Marine Microbiota

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Effect of Silicate Grain Shape, Structure, and Location on the Biomass and Community Structure of Colonizing Marine Microbiota

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Microbiota colonizing silica grains of the same size and water pore space, but with a different microtopography, showed differences in biomass and community structure after 8 weeks of exposure to running seawater. The absence of surface cracks and crevices resulted in a marked diminution of the total microbial biomass measured as lipid phosphate and total extractable palmitic acid. With increasing smoothness of the sand grain surface, examination of the community structure showed a marked decrease in procaryotes and algal microeucaryotes, with a relative increase in microeucaryotic grazers. A comparison of the colonizing sediment incubated in running seawater or at 32 m on the sea floor with a sediment core showed a decreased bacterial biomass with a different community structure and a decreased total microeucaryotic population of both grazers and algae. The quantitative differences in microbial biomass and community structure between the microcosms and the actual benthic population in the core were determined.

In a series of experiments designed to test the impact of oil and gas packing fluids on the marine benthic environment, the effects of the microtopography of sandy sediment grains were examined, using biochemical measures of microbial biomass and community structure. The provision of surfaces that are nonbiodegradable stimulates microbial growth and activity with a "bottle effect" by nutrient concentration (43), particularly where the water column nutrient concentration is low (10, 17). In soils and sediments, the great preponderance of the microbial biomass is on surfaces and not in the water phase (12, 21). In the soil, surface effects can account for changes in sensitivity to ultraviolet light, antibiotic action, or bacteriophage activity (20). Surface effects change nutrient distribution, degradative enzyme activities, and water concentrations, which in turn control the localization and community structure of the soil microbiota (4, 15, 30). Direct examination of the particles of soil or sediment shows a markedly heterogeneous distribution, with microbes occupying between 0.02 to 2% of the surface area (1, 16, 31). The attachment of the microbes involves chemotaxis, reversible and then irreversible binding with extracellular polymer production (11, 22), and often selection for microbes with specific attachment components (26, 27). With attachment, consortia of microbial species can create microzones detected as diverse shapes in fluorescent-antibody-staining microcolonies (33) or localized zones of anaerobiosis detected by tetrazolium reduction (27, 28).

In sandy sediments, like those used in these experiments, decreasing the grain size increases the respiratory activity of the microbes (14), and surface crevices protect attached microbes from mechanical abrasion and foster accumulation of microbes (23). Sieburth (29) and Weise and Rheinheimer (34) showed the concentration of bacteria and diatoms in the microdiscontinuities of marine sand grains by scanning electron microscopy. Weise and Rheinheimer (34) showed most of these organisms resisted removal from the surface when exposed to ultrasonic vibration.

In the present study, the analysis of lipid extracts is utilized to define the effect of sand grain microstructure and the location of the exposure to seawater on the microbial biomass and community structure. Some of these biochemical methods have been used to show the effects of changing surface chemistry on detrital microbiota and its associated macrofauna (42) or the effects of biodegradability of natural pine needles contrasted with similarly shaped needles of polyethylene (2).

MATERIALS AND METHODS

Materials. Solvents were purchased as distilled in

glass (Burdick and Jackson, Muskegon, Mich.). Chloroform (Mallinckrodt, St. Louis, Mo.) was freshly distilled in the laboratory. Other materials were as described (3).

Samples. A series of Plexiglas troughs (10 by 40 by 12 cm in depth) were filled to a depth of 5 cm with silicate substrates and exposed to unfiltered seawater (salinity, 35‰; temperature, 20 to 21°C) which was pumped from a depth of 26 m to a gravity-fed headbox that maintained a 200-ml/min flow rate over the sediments. The apparatus was developed by Hansen (13) and modified for exposure to oil and gas packing fluids as described by Cantelmo et al. (5). The troughs were housed on U.S. Naval Stage 1 platform 12 miles south of Panama City, Fla., 30°7.5′ N, 85°46.3′ W, in the Gulf of Mexico, between 1 August and 27 September 1978. The platform is in water 32 m deep. The light intensity and spectrum from the fluorescent lighting in the laboratory were adjusted to those on the ocean floor with a Plexiglas lid covered with blue plastic sheeting. Seabed controls consisted of plastic troughs set in weighted wooden frames placed on the bottom adjacent to the platform and of cores (each 5.08 cm in diameter and 15.2 cm deep) taken just before analysis from the sand near the platform but 20 m from any structure.

The troughs contained either glass beads with a 35 to 325 U.S. standard sieve size (45 to 500 μm in diameter), sand from the beach near Santa Rosa sound, Pensacola, Fla., or sand dredged from the bottom near the platform. The sand was dried, bleached in the sun, and then added to the troughs.

After 8 weeks of exposure to running seawater or the sea at the bottom, 10 2.54-cm-diameter sediment cores were removed from each trough and washed through a 1-mm sieve to recover the macrofauna. The seawater was decanted, and the sediment was quick frozen in plastic bags at −70°C. In the laboratory, 40-g (wet weight) frozen samples were recovered from the center of the samples, and the frozen sediment plus the pore water were extracted for lipid analysis.

Water content of the sediments. The water content of the sediment was determined by allowing the sediment to settle by gravity in a plastic beaker for 10 to 15 min after screening to remove macrofauna. The water was then decanted, and the sediment was frozen at −70°C with dry ice. To determine the water content, the frozen sediment was cracked open with a hammer, and then a sample was taken from the center and weighed while frozen and after drying at 105°C to constant weight.

The potential space remaining after centrifugal packing was determined by suspending 1 g (dry weight) of sediment in 2 ml of distilled water, centrifuging at 12,000 × *g* in a swinging bucket centrifuge rotor for 30 min, and measuring the proportion of the original suspension occupied by the sand.

Extraction. The sediments were extracted by the modified Bligh and Dyer chloroform-methanol extraction (3, 40), and the chloroform phase was filtered to remove water, dried under vacuum, and stored at −20°C under nitrogen until used.

Acid methanolysis. The fatty acid methyl esters were liberated from the lipids by mild acid methanolysis (in anhydrous methanol-concentrated HCl-chloroform, 10:1:1, vol/vol) in a test tube screw capped with a Teflon-lined cap at 100°C for 1 h. Equal volumes of both chloroform and water were added, and the solution was mixed with a Vortex mixer for 5 min and then centrifuged for 5 min. The water was removed for carbohydrate analysis, and the chloroform surface was washed without mixing as described previously (3). The solvent was removed, and the lipid components were fractionated by thin-layer chromatography.

Thin-layer chromatography. The lipids were partitioned on silica gel G (Whatman K6, silica gel 40Å, 250-μm thickness) with ascending chromatography in a solvent of petroleum ether-ethyl ether-acetic acid (80:20:1, vol/vol), the fatty acid methyl ester band (R_f, 5.5 to 6.5) was removed, and the esters were recovered. This procedure is quantitative (3).

Gas-liquid chromatography. Fatty acid methyl esters were fractionated on a 50-m glass capillary Silar 10C (phenyl and cyano alkyl polysiloxane) open tubular column with helium carrier gas at 1 ml/min in a Varian 3700 gas chromatograph with the CDS 111 data system. The samples were introduced in 2-μl amounts via the autosampler, with the system operating in a splitless mode (0.5-min venting time). A temperature program of 42 to 162°C, with a linear increase at 2°C/min, followed by a 30-min isothermal period, and a second increase at 1°C/min to 192°C that was maintained until the components were eluted, was utilized.

Identification of the fatty acid methyl esters. The fatty acid methyl esters were identified by their retention volumes before and after catalytic hydrogenation (39) on polar (Silar 10C) and nonpolar (OV-101) capillary columns. Major component identifications have been confirmed by gas chromatography-electron impact mass spectrometry with the Hewlett Packard 5995 system and by chemical ionization fragmentation with the Hewlett Packard 5785 system (3).

Fatty acid methyl ester nomenclature. The fatty acid methyl esters are designated as the number of carbon atoms:number of double bonds, with suffixes of a for anteiso and i for iso branching and with Δ for cyclopropanes. The position of the double bond nearest the omega end of the molecule is then given.

Lipid phosphate analysis. Lipids digested in 23% perchloric acid at 200°C for 2 h were determined colorimetrically (40).

Scanning electron microscopy. Samples were fixed in 0.175% glutaraldehyde (Ladd Research Industries, Burlington, Vt.) in filtered seawater at 4°C for 1 h and then transferred to 3.3% glutaraldehyde for 1 h more, washed, fixed in 1.33% osmium tetroxide in 0.2 M *sym*-collidine buffer, pH 7.4, at 4°C for 1 h, and washed with water. Samples were dehydrated in ethanol-water, critical-point dried, mounted, coated with gold, and examined in a Cambridge Stereoscan S4-10 microscope as described previously (24).

RESULTS

Surface morphology of the sediment grains. Scanning electron micrographs of the silica particles show the distinctive shapes (Fig. 1). The glass beads have a smooth surface and

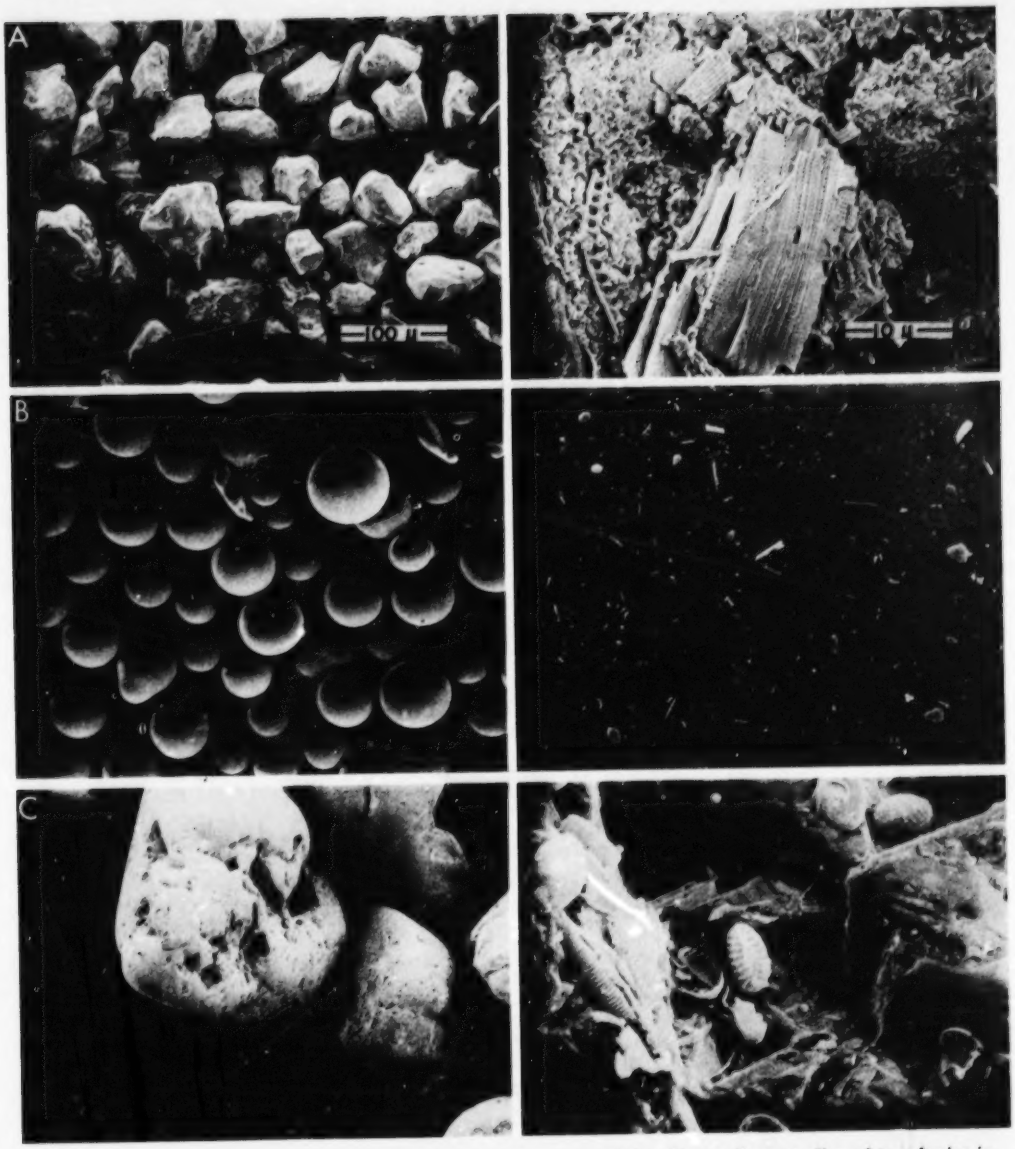

FIG. 1. *Surface morphology by scanning electron microscopy of the silica sediments allowed to colonize in running seawater. Sediment recovered from a depth of 32 m from the Gulf of Mexico (A); glass beads with diameters between 45 and 500 µm (B); sand recovered from the beach at Santa Rosa sound (C). The left column was magnified 50 times, bar represents 100 µm; the right column was magnified 2,000 times, bar represents 10 µm.*

a very sparse microflora (when examined at high power) (Fig. 1B). Santa Rosa beach sand shows rounded edges with surface irregularities that shelter a diverse microflora, as shown in the higher magnification (Fig. 1C). The irregular grains from the sea floor show a morphologically diverse microbial population when viewed at higher magnification (Fig. 1A). There was essentially no visible difference between the sand taken from the bottom, whether exposed on the

surface in flowing seawater, exposed in troughs on the sea bottom, or taken from the sedimentary core.

Pore space of the sediments. The weight loss on drying frozen sediments at 105°C to constant weight was 21.4 (1.4)% $\bar{x}$ (standard deviation [SD]) with no significant difference among the glass beads, Santa Rosa beach sand, marine sediment from troughs and benthic cores. After disturbing the sediment by suspending 1 g in 2 ml of water and then packing it by centrifugation, the proportion of the volume occupied by the sediment was: glass beads, 30.72 (0.9%); Santa Rosa beach sand, 30.73 (1.2)%; sediment from the underwater control, 31.56 (1.0)%; and marine core sediment, 30.07 (0.2)% [$\bar{x}$ (SD), $n = 4$]. The water pore space found in the sediments after experiments or after suspension and centrifugation was not different for the various types of sediments or experimental locations.

Effects of sediment grain structure on microbial biomass. Biomass and community structure measures with the lipids of the sedimentary microbiota are listed in Tables 1 and 2. Phospholipids measured by lipid phosphate increase progressively as the grain shape becomes more irregular and increase further in sediment exposed on the sea floor. This is true for the short-branched fatty acids characteristic of bacteria (a + i 15:0) and the product of the bacterial anaerobic monounsaturated pathway (18:1ω7). The polyunsaturated α-linolenic acid series typical of algae, such as 20:5ω3, increases progressively just as the bacterial components. This same pattern, with the exception of a high value in the beach sand, is typical of the γ-linolenic acid series, such as 20:4ω6. The total polyenoic fatty acids reflecting the total microeucaryotes are higher in the sands than in the glass beads.

Effect of sediment grain structure on the microbial community structure. The proportions of the fatty acids give insight into the microbial community structure (Table 2). The proportions of the a + i 15:0 fatty acids compared to the 15:0, which is a measure of a portion of the bacterial benthic components (3), are significantly lower in the glass beads than in the sands incubated in flowing seawater (columns 2 and 4, Table 2). The underwater control and the benthic core are both significantly enriched in this component of the population. The *cis*-vaccenic acid-forming bacterial portion of the procaryotic community is significantly lower in the rounded glass beads and Santa Rosa beach sand than in the control sand, whether measured as a proportion of the total lipid (18:1ω7/16:0) or the oleic acid-containing procaryotes and eucaryotes (18:1ω7/18:1ω9). Both these ratios are higher in the lipids from organisms in the benthic cores (column 6, Table 2). The proportion of 18:2ω6 (linoleic acid) found in fungi, blue-

TABLE 1. *Biomass and community structure of the sedimentary microbiota colonizing the silica surface*

Component	Glass beads[a]	Santa Rosa sand[b]	Control sand[c]	Underwater control[d]	Sedimentary core[e]
Lipid phosphate[f]	47.3 (6.0)***[g]	88.0 (41.0)**	252.0 (39.0)	359.0 (72.0)***	586.0 (72.0)***
Total 16:0[f]	12.6 (1.3)***	39.0 (9.0)*	54.0 (16.0)	81.0 (37.0)***	78.0 (31.0)**
Ratio lipid P/16:0	3.57 (0.01)	2.29 (1.45)**	5.59 (2.83)	4.44 (4.2)	9.36 (5.81)*
a + i 15:0[f]	0.4 (0.4)***	2.0 (1.0)***	7.2 (3.0)	12.7 (6.7)**	14.8 (8.5)***
15:0	0.7 (0.5)***	2.0 (0.9)***	7.5 (3.1)	9.55 (4.32)	6.5 (2.2)
Δ17:0 + Δ19:0	0.5 (0.1)*	3.0 (2.0)	5.0 (4.2)	3.98 (2.28)	2.5 (0.7)
18:1ω7	0.7 (0.4)***	3.3 (0.9)***	12.3 (4.7)	19.8 (8.1)***	23.8 (8.5)***
18:1ω9	2.0 (0.5)**	8.0 (3.4)	11.8 (7.7)	14.95 (8.5)	14.4 (7.1)
18:2ω6	0.5 (0.3)*	2.1 (2.0)	3.0 (2.0)	2.62 (1.54)	1.8 (1.5)
20:4ω6	4.0 (3.0)	9.0 (6.0)**	3.8 (3.0)	6.74 (2.5)***	10.7 (2.2)***
20:5ω3	0.5 (0.7)	1.2 (1.0)	1.7 (1.7)	2.37 (2.6)	3.7 (4.1)
24:0	0.5 (0.2)**	2.2 (3.0)	3.2 (1.8)	3.03 (1.34)	2.8 (0.8)
22:5ω3	— —[i]	— —	— —	0.26 (0.57)	— —
22:6ω3	— —*	0.7 (1.0)	0.7 (0.5)	0.68 (0.48)	1.0 (0.9)
Total polyenoics[h]	6.0 (4.0)	13.6 (10.0)	9.7 (4.4)	12.1 (6.99)*	17.8 (8.6)**

[a] Glass beads. See Fig. 1B; (SD), $n = 3$.
[b] Santa Rosa sand. See Fig. 1C; (SD), $n = 5$.
[c] Control sand. See Fig. 1A; (SD), $n = 15$.
[d] Underwater control. (SD), $n = 30$.
[e] Sedimentary core. (SD), $n = 5$.
[f] nmol/40 g (wet weight) (31.5 g [dry weight]) of sand for all fatty acids listed.
[g] *, **, ***, Significant differences between means of the control sand column and other columns at the 0.1, 0.05, and 0.01 level, respectively, using a one-way analysis of variance.
[h] Total polyenoics 18:2ω6, 20:4ω6, 20:5ω3, 22:5ω3, 22:6ω3.
[i] —, <0.01 nmol/40 g (wet weight).

TABLE 2. *Community structure of the sedimentary microbiota colonizing marine silica surfaces*

Fatty acid ratios	Glass beads[a]	Santa Rosa sand	Control sand	Underwater control	Sedimentary core
a + i 15:0/15:0	0.55 (0.56)**	1.02 (0.23)	0.98 (0.06)	1.32 (0.28)***	2.64 (0.21)***
a + i 15:0/16:0	0.05 (0.04)***	0.06 (0.03)***	0.15 (0.03)	0.17 (0.04)	0.21 (0.08)**
Δ17:0 + Δ19:0/16:0	0.05 (0.01)***	0.07 (0.04)*	0.09 (0.02)	0.05 (0.02)***	0.04 (0.01)***
18:1ω7/16:0	0.07 (0.04)***	0.10 (0.02)***	0.26 (0.03)	0.28 (0.04)	0.36 (0.04)***
18:1ω7/18:1ω9	0.32 (0.09)***	0.47 (0.23)***	1.31 (0.4)	1.47 (0.45)	1.79 (0.41)**
18:2ω6/16:0	0.05 (0.03)	0.07 (0.06)	0.05 (0.02)	0.03 (0.01)**	0.02 (0.02)*
20:5ω3/16:0	0.08 (0.08)	0.04 (0.04)	0.05 (0.04)	0.03 (0.02)**	0.05 (0.05)
20:4ω6/16:0	0.40 (0.3)***	0.23 (0.11)***	0.05 (0.04)	0.08 (0.03)	0.17 (0.05)***
22:5ω3/16:0	— —	0.01 (0.02)	— —	0.001 (0.002)	0.004 (0.004)
22:6ω3/16:0	— —**	0.02 (0.02)	0.02 (0.008)	0.01 (0.007)	0.011 (0.01)
Total polyenoics/16:0	0.54 (0.42)***	0.39 (0.17)***	0.17 (0.04)	0.18 (0.07)	0.26 (0.06)***
ω6/ω3 polyenoics	6.67 (0.87)***	6.30 (4.23)***	1.0 (1.1)	3.4 (2.2)***	10.9 (18.3)*

[a] Footnotes *a* through *e* and *g*, *h*, and *i* of Table 1 apply to Table 2.

green algae (cyanobacteria), green algae, and some protozoa as well as filamentous bacteria (3, 32, 38) is higher in the sediments exposed to running seawater than in the underwater control or benthic core. The proportion of algal polyenoic 20:5ω3/16:0 is essentially the same in all the sediments, but the proportion of the microfaunal protozoal polyenoic fatty acid 20:4ω6/16:0 is significantly higher in the glass beads and rounded Santa Rosa beach sand than in the disturbed sediments exposed to running seawater or incubated under the sea (columns 2 through 5, Table 2). The benthic core is richer in this γ-linolenic acid series component than the disturbed marine sands, but less so than the rounded sand or glass beads.

The total polyenoic fatty acids (18:2 to 22:6) are lowest in the glass beads and highest in the benthic core. The microfaunal to microfloral ratio (the polyenoic ω6/ω3 ratio) is greatest in the undisturbed core and higher in the rounded sands and glass beads than in the control sand.

DISCUSSION

Biochemical measures of microbial biomass and community structure. Over the last several years a suite of biochemical measures has been applied to measurement of the biomass and community structure of natural microbial populations. The measurement of extractable phospholipid by lipid phosphate has been shown to correlate well with assays of microbial biomass and activity such as total adenosine, lipid and deoxyribonucleic acid synthesis, enzymatic activities, and the muramic acid and glucosamine recovered in hydrolysates (7, 9, 19, 35, 36, 37, 40). Phospholipids are particularly enriched in procaryotes (18), so this measure appears weighted towards the bacteria in microbial populations. Palmitic acid (16:0), which is probably the most ubiquitous of all the fatty acids (8, 18), is a measure of the total lipid, which includes the endogenous storage natural waxes and fats as well as the phospholipids. The microeucaryotes contain much more of these neutral lipids and thereby a smaller proportion of phospholipids and so may be estimated by the ratio of total lipid to phospholipid (8, 18).

The community structure of the microbiota can be estimated with a detailed analysis of the lipid-derived fatty acids (3). From the literature (3, 18, 38, 41) various fatty acids can be associated with particular components of the microbial population. The bacteria contain high concentrations of a + i 15:0, 15:0, Δ17:0, Δ19:0, and 18:1ω7. The fungi, cyanophytes, and flexibacteria contain 18:2ω6. Polyenoic fatty acids longer than 20 carbon atoms and fatty acid alkyl longer than 22 carbon atoms are found in microeucaryotes. Among polyenoic fatty acids, the α-linolenic (ω3) acid series of polyenoics is found largely in algae, and the γ-linolenic (ω6) acid series of polyenoics is found in protozoa and micrometazoa (3). Validation of these assignments has come from analysis of artificial mixtures of microbes, manipulation of detritus with cultural conditions and antibiotics, with comparisons between activities, analyses and scanning electron microscopic morphologies, and by the analysis of the effects of selective predation of the sedimentary microbiota by the sand dollar *Mellita quinquiesperforata* (3, 38, 41).

Sand grain microtopography and microbial population structure. In sand grain microcosms exposed to running seawater with the same water pore content and with grains of the same size, smoothness results in a marked decrease in the total microbial biomass measured as the lipid phosphate and extractable palmitic acid. The bacteria are decreased as reflected in the lipid phosphate and the absolute amounts of a + i 15:0, 15:0, Δ17:0 and Δ19:0, and 18:1ω7, as well as in the relative amounts of these fatty acids in the extracted lipids. The microeucar-

yotes are also somewhat decreased with the glass beads, as measured by the decreased lipid phosphate/total 16:0 ratio and the total polyenoic fatty acids. The algal component of the microeucaryotes measured as the ω3 polyenoic acids appears decreased, and the relative proportion of the grazers measured by 20:4ω6 is increased as the smoothness of the surface increases. Santa Rosa beach sand with rounded edges and cracks and crevices showed higher microeucaryotic population reflected in the total polyenoics and 24:0 than did the smooth glass beads.

Effect of the location on colonization of angular sand grain sediment. The angular sand grains incubated at the surface in flowing seawater show a different microbial population than the same type of grains incubated on the sea bottom at 32 m. The running seawater microcosm supported a smaller bacterial assembly with a statistically significant higher proportion of cyclopropane fatty acid-containing bacteria and linolenic acid (18:2ω6)-containing microbes than did the same sand colonized on the sea bottom. Absolute amounts of all other fatty acids were increased in the sand incubated on the sea bottom. The microeucaryotes of both the ω3 and ω6 polyenoic fatty acid types were higher in disturbed sand incubated on the bottom, where more direct colonization from the surrounding sediment was possible.

Possible causes for differences in sediment community structure. Surface abrasion, which was minimal in the running seawater tanks but possible in the sediment and tanks incubated on the sea floor, seems unlikely to have been responsible for the microbial community structure changes, since the bottom sediments supported the richest microbial assemblies (Table 1). Surface abrasion can decrease the total sedimentary microbial biomass (23).

Bacteria are the primary colonizers of new surfaces in marine environments (6, 11, 24), yet they are depressed where shelter in the form of surface irregularities is missing in sediments incubated with raw seawater for 8 weeks. This strongly suggests that grazing is modifying the microbial community structure. In studies utilizing these methods, amphipod grazing has been shown to markedly shift the composition of the microbial community, as evidenced by changes in morphological appearance and increases in activity and biomass (25). The correlation of highest relative proportions of 20:4ω6 (found in bacterial grazers such as protozoa and nematodes) with lowest relative proportions of bacterial fatty acids helps confirm these speculations. The absence of some predators of the microbial grazers in the colonizing troughs as compared with the samples incubated on the bottom and the cores could explain the high rates of bacterial predation, particularly where the protection in the lee of surface irregularities is absent. Placing the trays on the sea bottom concentrated a major predator of the grazers. After a week, a single octopus (*Octopus vulgaris*) was living in a burrow under each set of five trays. They and other large animals were observed at night by scuba divers processing the sand in the trays.

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